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(54) Title: ARTEMISIA ANNUA TRANSFORMED WITH AGROBACTERIUM RHIZOGENES

(57) Abstract

A method of transforming *Artemisia annua* with *Agrobacterium rhizogenes*. More particularly, a method for producing an *Agrobacterium rhyzogenes* hairy root cell culture of *Artemisia annua*. The method of transformation of *A. annua* includes the transformation of whole plants with *A. rhyzogenes*, and also includes transformation of *A. annua* root cultures with *A. rhizogenes*. The present invention also relates to transformed *A. annua*. The present invention also relates to a method for extracting enhanced amounts of a plant secondary metabolite from plant tissue with limited loss of tissue viability by reversibly permeabilizing the tissue membrane is disclosed.

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ARTEMISIA ANNUA TRANSFORMED WITH AGROBACTERIUM RHIZOGENES

Background of the Invention

Plants grown in vitro can provide a major source of specialty chemicals which are plant secondary metabolites. For example, artemisinin, a terpenoid found in the herb, Artemisia annua, is a promising therapeutic for treatment of malaria. Artemisinin has also been shown to be effective against *Pneumocystis carinii*, an opportunistic pathogen often associated with immune suppression and AIDS.

Approximately one half of the world's population is currently at risk of contracting malaria. It is estimated that there are more than 200 million cases, and 1-2 million deaths each year attributable to this disease. The most prevalent form of malaria is caused by *Plasmodium falciparum*, which is responsible for the deaths each year of over one million children under five years of age. Despite attempts during the 1950s to eradicate the disease, the incidence of malaria has risen since 1960 in many areas of the world, in part because of the spread of multi-drug resistant strains of *Plasmodium falciparum*. As a result, the need for practical and effective anti-malarial drugs is as great as ever.

The herb, *A. annua*, produces a compound, artemisinin (AN), which has been shown to be a promising therapeutic. However, this highly effective compound is produced by native plants in minute quantities, and by tissue cultured plants at levels significantly less than the best native

plants. Efforts to obtain higher production levels in ither native plants or their cultured tissues would make therapeutic use of artemisinin on a large scale, a reality.

Except for shikonin and berberine, there has been little success in the profitable production of secondary metabolites from plant cultures. The lack of success is due in part to the fact that these chemicals are present only in small amounts within the plant. In addition, in vitro and in vivo cloning of plants, especially woody plants, is extremely difficult because of slow growth rates, reduced or lack of rooting ability, frequent systemic microbial contamination, phenotypic instability, and phenolics build-up. Micropropagation is also very difficult.

There has been some success in the establishment of undifferentiated callus and cell culture lines for production of secondary metabolites. (See, for example, U.S. Patent No. 5,019,504 entitled, "Production of taxol or taxol-like compounds in cell culture," by Christen et al.) However, secondary metabolism is frequently linked to differentiation. Therefore, most undifferentiated cultures are not useful for producing secondary metabolites. Thus, there is a need to develop fast-growing differentiated plant cultures for the production of artemisinin.

Summary of the Invention

Th subject invention relates to a method for introducing at l ast a portion of Ri plasmid DNA of Agrobacterium rhizogenes into Artemisia annua. The present invention further relates to a method of producing transformed hairy root cultures. The subject invention relates more particularly to a process for producing an *A. rhizogenes*-transformed hairy root culture of *A. annua*. The process of transformation of *A. annua* includes infection of *A. annua* whole plants with *A. rhizogenes*, as well as infection of *A. annua* axenic root cultures with *A. rhizogenes*.

The present invention also relates to transformed *A. annua* root cultures and *A. annua* plants, produced by infection and transformation of *A. annua* roots or whole plants by *A. rhizogenes*.

The subject invention also relates to a process for extracting enhanced amounts of artemisinin from transformed root cultures of *A. annua*, with limited loss of tissue viability, by reversibly permeabilizing the plant tissue membrane. The process includes three steps: 1) destabilizing root tissue membrane to effect partial release of artemisinin; 2) removing released artemisinin to enhance the diffusion gradient and thereby increase artemisinin efflux; and 3) restabilizing the root tissue membrane to inhibit further secondary metabolite release.

Brief Description of Drawings:

Figure 1 is a bar graph depicting hairy root growth resulting from application of an *A. rhizogenes* culture to wounds in various parts of *A. annua* plants.

Figure 2 is a bar graph depicting hairy root growth resulting from injection of an *A. rhizogenes* culture into various parts of *A. annua* plants.

Figure 3 is a high performance liquid chromatogram of artemisinin extracted from hairy roots produc d by *A. annua* strain YU transformed with *A. rhizogenes* strain ATCC number 15834.

Figure 4 is a high performance liquid chromotogram of artemisinin extracted from hairy roots produced by *A. annua* strain AR transformed with *A. rhizogenes* strain ATCC number 15834.

Figure 5 is a high performance liquid chromatogram of an artemisinin standard.

Figure 6 is a high performance liquid chromatogram of an opine extract of hairy roots produced by *A. annua* strain YU, showing the presence of mannopine.

Figure 7 is a high performance liquid chromatogram of an opine extract of hairy roots produced by *A. annua* strain AR, showing the presence of mannopine.

Figure 8 is a high performance liquid chromatogram of a mannopine standard.

Detailed Description of the Invention

Differentiated tissues (e.g. roots) and especially transformed roots are known to be genetically stable and often produce high levels of secondary metabolites. We have now shown that artemisinin can be produced in significant quantity in root cultures, that *A. annua* can be genetically transformed, that transformed root cultures contain artemisinin, and that a variety of environmental factors can be manipulated to increase the artemisinin content in whole plants. Therefore, it appears that root cultures of *A. annua*, especially transformed roots, can be manipulated in their growth environment to yield a stable tissue system for the production of artemisinin at high levels and at reasonable production rates. Transformed roots generally grow 5-10 times faster than untransformed roots, and typically demonstrate proportional increases in

the production rate of secondary metabolites. These roots are also genetically stable over a number of years.

Described herein is work demonstrating that *A. annua* has been transformed with *A. rhizogenes* and resulted in production of hairy roots.

As described in the Examples, it has been shown that it is possible to transform *A. annua* with Agrobacterium rhizogenes. Transformation results from the transfer from the bacteria to the plant of at least a portion of the Ri plasmid DNA; generally, less than the entire plasmid will be stably transferred to the plant, but the portion transferred must be sufficient to cause transformation. We have demonstrated transformation in whole plants, as well as in root cultures, both nonsterile and sterile. Although transformation as described in the present application was accomplished by infection with Agrobacterium rhizogenes, ATCC strain 15834, it is to be understood that other strains of Agrobacterium rhizogenes harboring a Ri plasmid may be used. The experiments described herein have been carried out using the following strains: WV (West Virginian), WRAIR (Walter Reed Army Institute of Research), AR (American Arboretum), YU (Yugoslavian) and Super Annie. However, these strains are used only as examples, and it is to be understood that transformation as described in the present application can be accomplished with other strains of *A. annua*.

The experiments described herein demonstrate transformation of *A. annua* plants, and *A. annua* sterile root cultures; *A. annua* sterile root cultures are a preferred vehicle for transformation. According to the method, a nonfatal wound is made on a part of an *A. annua* plant (e.g., stem, root, leaf, branch) and a culture of *A. rhizogenes* containing a Ri plasmid is applied. The *A. rhizogenes* culture would preferably be in late log phase or early stationary phase of growth, and can be applied

with a sterile applicator such as a toothpick, swab, loop or other suitabl means. Alternatively, the wound and application would occur simultaneously, as would be the case if a syringe or needle were used to apply the bacteria. In addition, a wound site could include a wound on tissue growing in in vitro culture. For example, sterile root cultures could be chopped or minced and, thereby, wounded before the bacterial culture is applied. Although *A. rhizogenes* ATCC strain number 15834 is used in the following examples to transform *A. annua*, it is reasonable to assume that other strains of *A. rhizogenes*, or other similar vectors, would also transform *A. annua*. For example, strain number 15834 is classified as a mannopine strain of *A. rhizogenes*, and so it is reasonable to assume that there exists another mannopine strain that can transform *A. annua*. After the plants have been wounded and contacted with the *A. rhizogenes* culture, they are maintained under conditions appropriate for growth. These conditions will vary depending on whether cells or roots in culture or entire plants are infected. Conditions for growth are described in the examples.

In order to obtain enhanced amounts of many plant secondary metabolites, it is preferred that the tissue is differentiated. Plant roots, including genetically transformed hairy roots, provide an especially preferred tissue for obtaining many plant secondary metabolites, including artemisinin. Generally, plant cultures which are growing in late exponential growth phase and stationary phase produce enhanced amounts of secondary metabolite.

According to the method of the subject invention, transformed *A. annua* hairy roots are grown under conditions appropriate for cell growth. A particularly useful method of obtaining plant tissue, which contains enhanced amounts of secondary metabolite, is by culturing

in a nutrient mist bior actor (NMB), as described in U.S. Pat nt Number 4,857,464 entitled "Mist Cultivation of Cells" by Weathers and Giles, the teachings of which are incorporated herein by reference. Cultivation of plant cells in a nutrient mist bioreactor offers the advantage of providing cells with a readily available gaseous and liquid nutrient supply. In addition, use of the bioreactor permits rapid change in culture conditions (e.g., nutrient or extractant addition) to allow for precise control of the culture environment. Further, in a bioreactor, cells are supported on screens within a sterile chamber which allows cell products and media to continuously drain away from the tissue into a collection chamber. In order to accommodate the growth of and extraction from transformed hairy roots, the Nutrient Mist Bioreactor should be modified to increase the mist volumetric throughput. This is easily accomplished by placing the ultrasonic transducer inside the culture chamber. In addition, a collapsible trellis can be placed in the culture chamber for use in scale-up. Both of these modifications are described in detail in the parent application, U.S. Serial No., 07/719,183, the teachings of which are incorporated by reference.

Artemisinin can be extracted from the transformed roots by a variety of means. For example, the transformed hairy root membranes can be destabilized to effect partial release of artemisinin from the tissue. Destabilization of plant tissue membranes can be accomplished by any of a number of techniques, performed alone or in combination. For example, the plant tissue membrane can be exposed to elevated temperatures for various periods of time. Culturing plant tissue in temperatures in the range of 25°-45°C for times ranging generally from 1 minute to 2 hours is useful for obtaining partial release of most

secondary metabolites, while maintaining plant tissue viability.

Alternatively, destabilization of plant tissue membranes can be accomplished by contacting the plant tissue with a permeabilizing agent at an appropriate temperature and for an appropriate length of time. Destabilization can also be accomplished by excluding membrane stabilizers from the culture environment.

Subsequent to destabilizing the plant membrane, solvents can be added to plant culture medium to effect greater extraction of secondary metabolites. For example, artemisinin is a nonpolar compound and is not soluble in aqueous solutions, such as the culture medium. Therefore, a nonaqueous solvent, which does not significantly decrease the plant tissue viability, can be added to the plant tissue surroundings (e.g., the culture medium) to enhance extraction of artemisinin. Alternatively, the permeabilizing agent itself can be a nonaqueous solvent. Preferred nonaqueous solvents include: ethanol, 65% and 75%, polyethylene glycol (PEG-400), tomatine, poly-L-Lysine, 50% Cremaphor EL in a short chain alcohol (e.g., methanol), DMSO, Triton X-100, Brij, Tween-80 and cumene peroxide. Ethanol and 50% Cremaphor EL in methanol or in any other short chain alcohol are particularly useful for extracting taxol from Taxus species. Alternatively, the permeabilizing agent itself can be a nonaqueous solvent.

In the next step of the subject method, artemisinin is removed from the plant tissue surroundings. For example, removal of the released secondary product can be accomplished by exchanging the medium containing the released product with fresh medium. Rapid exchange of medium results in near continuous removal of released product, thereby increasing secondary metabolite release.

In the final step of the method of the invention, the plant tissue membrane is restabilized to inhibit secondary

metabolite release and to enhance plant tissue viability. Restabilization of plant tissue membranes can be accomplished by any of a number of techniques, performed alone or in combination. One approach is to remove the condition which promoted destabilization. For example, the temperature of the culture medium can be cooled (e.g., to room temperature). Alternatively, the destabilizing agent can be removed. Further, divalent cations can be introduced into cultures which were destabilized by being cultured in medium which lacked divalent cations, or other plant cell membrane components which contained a permeabilizing agent. Addition of sterols or other components of cell membranes can also effectively restabilize plant cell membranes and enhance plant tissue viability.

The disclosed methods for artemisinin release can be applied to a viable culture without significant loss of biomass. This biomass is therefore conserved and available for further permeabilizations to obtain product release without the need to accumulate more biomass, which could require weeks to accomplish in a large scale bioreactor. Therefore, the cost of processing large amounts of biomass in batch production is effectively reduced. In addition, the degree of selectivity toward specific products offered by the choice of destabilization methods provides additional control over process development.

Possible extraction method would involve contacting the transformed roots with compounds, for example derived from fungi, which induce secondary metabolite production. One such compound is chitosan, a cell wall component derived from a common soil fungus, *Mucor rouxii*. According to this method, a late exponential phase or stationary phase transformed root culture is preferred. Approximately 1 mg of fungal chitosan/g fresh cell weight

is added to the sterile root culture. Batch harvesting would take place within 48 hours after th introduction of th elicitor (e.g., chitosan). the exact timing of th harvest step as well as the amount of elicitor introduced into a culture will dependent on culture conditions as well as the individual strain used.

The following describes one approach to obtaining increased production of artemisinia from *A. annua* plants. The first step is to obtain sterile cultures of transformed roots of *A. annua* (for example, WV (West Virginian), WRAIR (Walter Reed Army Institute of Research), AR (American Arboretum), YU (Yugoslavian) and Super Annie) and then to use these transformed cultures to test two methods for speeding growth: CO_2 enrichment and use of nutrient mists. After optimizing for those two growth conditions, the kinetics of growth relevant to artemisinin production for transformed roots in both shak flasks and in nutrient mists will be measured. Finally the effects of the environmental parameters known to enhance production of artemisinin in whole plants (pH, temperature, water stress, and salinity) will be measured for their effects on artemisinin production in cultured roots.

As described herein, it has been shown that the antimalarial, artemisinin, is produced in significant quantity (3.26% of dry weight) by root cultures. We have also shown that a variety of environmental factors (light, salinity, and water stress) can be manipulated to increase the AN content in whole plants. We have further shown that *A. annua* can be transformed by *A. rhizogenes* to yield the phenotype hairy roots producing artemisinin. Differentiated tissues (e.g. roots) and especially transformed roots are known to be genetically stable and often high producers of secondary metabolites. Therefore,

it is possibl to manipulate root cultures of *A. annua*, especially transformed roots, in their growth environment to yield a stable tissue system for the production of artemisinin at high levels at a reasonable production rate.

Yet another advantage of the present invention is the use of Ri plasmid DNA as a genetic marker when transferred to *A. annua*. According to this method, hairy root clones are screened for artemisinin production (or production of artemisinin intermediates such as arteannuin B or artemisinic acid). If a clone does not produce the desired compounds, then it can be assumed that the portion of the Ri plasmid has interrupted the genetic coding sequence for production of the compound. (This effect was observed in transformed *Beta vulgaris* as non-pigmented cultures; in contrast, normal root cultures as well as most transformed cultures produced enough red pigment to be visible.) By scanning the plant genomic DNA for the Ri plasmid segment in transformed *A. annua*, the sites of secondary metabolite synthesis can be mapped. This procedure can be applied to artemisinin production in order to map a portion of or the entire synthetic pathway. Essential elements in the pathway can then be cloned, for example into yeast, thus enabling production rates to be even further increased.

The present invention will now be illustrated by the following Examples, which are not to be seen as limiting in any way.

Example 1 - Transformation of Axenic Root Cultures of Artemisia annua with Agrobacterium rhizogenes:

In this Example we describe the production of sterile root cultures from sterile germinated seedlings, and subs quent transformation of the root cultures by *A. rhizogenes*.

Se ds of *A annua* were germinated and cultured inside of plastic tents to maintain high humidity (≥ 90% RH). The sterile seeds were germinated on plate count agar then transferred to PlantCons (Flow Labs) containing White's basal salts (Sigma), pH 5.5-6.0 (plus 0.22% Gel-Rite (Sigma)).

The roots were excised and placed in White's medium (Sigma) containing 2% sucrose. These cultures have been maintained for about 2 years. The roots are subcultured by transferring about 1 gram fresh weight to 40 ml fresh medium plus 10 ml of spent medium from the previous culture.

Agrobacterium rhizogenes strain 15834 was obtained from the ATCC and prepared as per package directions for growth of freeze dried cultures. The culture was allowed to grow for 24-48 hours. The bacterial culture was then induced using acetosyringone (Aldrich) (0.039 g was dissolved in 1 ml 70% ethanol, then 0.4 ml of this solution was filter sterilized into 10 ml of bacterial culture plus 30 ml of fresh YMB broth). The induced culture was grown for 48 hours before infecting.

Roots were infected by dicing up the roots with a sterile scalpel and transferring the roots to either 10 ml fresh medium plus 1 ml induced bacterial culture, or to 40 ml of fresh medium with 0.2 ml of induced bacterial culture. The 10 ml culture was incubated for 1 hour and the 40 ml culture for 48 hours at 20-22°C.

The roots were then placed in liquid White's medium containing 250 mg/liter augmentin and cultured an additional 3-7 days. Next, the roots were removed from the liquid medium (aseptically), blotted dry and plated on White's solid medium containing 0.025% carbenicillin. After approximately 2 weeks on Whites medium with 250 mg carbenicillin/liter, the roots are dipped for 1-10 minutes

in 10% bl ach. Roots prepar d this way are free of bacteria.

Example 2 - Infection of Whole Artemisia annua Plants With Agrobacterium rhizogenes:

Seeds of *A. annua* (strains YU, WRAIR, AR, Super Annie and WV) were germinated and cultured inside of plastic tents to maintain high humidity (≥ 90% RH). The plants were grown until they had a minimum stem diameter of 1 mm prior to infection.

For infection of sterile plants, seeds were also surface sterilized using 10% commercial bleach plus 0.5 ml Tween 80/100 ml solution. The sterile seeds were germinated on plate count agar then transferred to PlantCons containing White's basal salts, pH 5.5-6.0 (plus 0.22% Gel-Rite). The sterile plants were grown for 4-6 weeks prior to infection using a 16 hour light cycle at 20-22°C.

The bacterial culture obtained from ATCC was prepared as per package directions for growth of freeze dried cultures, and allowed to grow for 24-48 hours. The bacterial culture was induced using acetosyringone (0.039 g was dissolved in 1 ml 70% ethanol, then 0.4 ml of this solution was filter sterilized into 10 ml of bacterial culture plus 30 ml of fresh YMB broth). The induced culture was grown for 48 hours before infecting whole plants.

Plants were infected by one of two methods: by injection of a bacterial suspension (10^8 cells/ml) into the stem, branch or apical meristem with a sterile needle or by making a tangential cut from 5-15 mm in length into the side of the stem, branch or leaf, and then swabbing the same bacterial suspension onto the fresh wounded surface. The plants were maintained at 20-27°C in a high

humidity (≥ 90%) environment to ncourage and to sustain hairy root formation and growth.

After the roots were excised, some were surface sterilized with 10% commercial bleach for up to 5 minutes, rinsed 3 times with sterile dH_2O, blotted dry and placed on White's medium with 2% sucrose, 0.0125% carbenicillin, 125 mg/liter augmentin and 0.22% Gel-Rite. Other roots were collected for verification of transformation.

Roots which formed at the site of infection of initially sterile plants were treated less harshly since contamination was due only to the Agrobacterium infection. These roots were placed on the same White's medium but without the augmentin. Final clean up of tissue occurs after large quantities of tissue are maintained in liquid culture with antibiotic. This process involves surface sterilization with commercial bleach (10% for up to 10 minutes) and/or using Alcide exspor.

Example 3 - Hairy Root Production:

Roots formed at the site of the infection of whole plants after 4-5 days. These roots became more numerous and larger as they continued to grow in the high humidity environment. After the roots were harvested (about 1 week), more hairy roots continued to grow at the original site of infection.

Figure 1 shows the results of application of the bacterial culture to wounds in different parts of the plant. These data are expressed as percent transformation as a function of site of infection. The data represent observed hairy root growth at the site of application 1-2 weeks after infection. The results demonstrate the application of *A. rhizogenes* to a wound site in the stem of *A. annua* results in very high rates of transformation.

Figure 2 shows the results of a similar experiment in

which th rout of infection was by injection, instead of by application to a wound. The results from injecting the bacterial culture are similar to the results observed when the bacteria are applied to a wound: injection into the stem caused the highest rates of transformation (between 80% and 100% transformation).

Example 4 - Opine Production by the Hairy Roots:

An assay for opines was performed on the hairy roots which resulted from application or injection of *A. annua* with *A. rhizogenes*. Opines are compounds that are not normally produced by plant tissues. These compounds are coded for in the bacterial plasmid although the bacteria do not produce opines. *A. rhizogenes* strain ATCC 15834 codes for the expression of two opines, agropine and mannopine.

Figures 6 and 7 are high performance liquid chromatograms of hairy root extracts prepared from *A. annua* strains YU and AR respectively.

Tissue extracts were prepared from hairy roots taken from YU and AR strains of *A. annua*. These extracts were prepared according to the method of J.L. Firmin, J. Chromatography, 514:343-347 (1990). High performance liquid chromatography was performed on the hairy root extracts to detect the presence of opines in the tissue extracts (Firmin (1990)).

Mannopine, obtained from Sigma Chemical Co., was used as a standard (0.5 μg was injected) (Figure 8). The assay used in this experiment is sensitive to picomole quantities of opines. The full scale output of the chart recorder is 10 volts. The small peaks in the standard chromatogram (Figure 8) are impurities in the standard. The second large peak is mannopine. The residence time for mannopine in the standard (16.4 minutes) matches similar peaks in the tissue extracts from hairy roots

taken from the YU and AR strains. Spiking the tissue extracts with the standard solution (a typical verification method) produced larger peaks in the tissue extracts.

The chromatograms of tissue extracts of hairy roots from strains YU and AR revealed the presence of opines (Figures 6 and 7), thereby confirming transformation of these plants by *A. rhizogenes*.

Example 5 - Artemisinin Production by Hairy Roots:

Artemisinin production by the transformed hairy roots was demonstrated by a new, one step method for separating and quantitating artemisinin. Previous methods for artemisinin extraction and detection involved numerous and lengthy solvent extractions and a time consuming HPLC step requiring 40-50 minutes to verify purification. However, the method described herein involves only one brief solvent extraction step, and a brief centrifugation step. According to this method, plant tissue containing artemisinin is ground in the presence of ethanol, then the cell debris is removed by centrifugation. For example, the tissue can be ground in a mortar and pestle, or similar device, and the artemisinin extracted using 70% ethanol. According to the new method for purifying artemisinin, hairy root tissue (40 mg fresh weight) from strains AR and YU was ground with a mortar and pestle in 0.8 ml 70% ethanol. The tissue was then centrifuged at 10,000 x g for 10 minutes and the supernatant was filtered through a 0.2 micron filter. Twenty µl of the supernatant was injected onto a 25 cm C18 column (Rainin) with 5 µ beads fitted with a 5 cm guard column of like material. The mobile phase was 80% acetonitrile and 20% water, and the flow rate was 1 ml/min. Detection of artemisinin was by absorption of UV light at 214 nm. Figure 3 is a chromatogram of an xtract of hairy roots from transformed

A. annua strain YU. Figur 4 is a chromatogram of an xtract of hairy roots from transform d *A. annua* strain AR. Figure 5 is a chromatogram of an art misinin standard. The chromatograms show the presence and distinct separation of artemisinin in the hairy roots from transformed strains YU and AR.

Example 6 - Transformed Axenic Root Cultures

Axenic root cultures infected with *A. rhizogenes* ATCC strain 15834 produced hairy roots. These roots began to form approximately 2 weeks after the original infection, and displayed a characteristic transformed phenotype. The typical phenotypic differences between hairy roots and normal roots were observed. Phenotypic differences between normal controls and transformed root cultures were assessed qualitatively. The phenotypic changes exhibited by the transformed root cultures included: 1) lack of geotropism, 2) prolific root hairs, and 3) increased growth rate. The hairy roots approximately doubled in size after 2-3 days, while little growth was detected in the non-infected cultures during this same time period.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method of transforming *Artemisia annua* with *Agrobacterium rhizogenes*, comprising infecting an *A. annua* strain with *Agrobacterium rhizogenes* containing a Ri plasmid, thereby producing *Artemisia annua* containing Ri plasmid DNA.

2. A method of Claim 1 wherein *Artemisia annua* is infected by introducing the Ri plasmid into a wound site on *Artemisia annua* by contacting the wound site with an *Agrobacterium rhizogenes* containing the Ri plasmid.

3. A method of Claim 2 wherein the wound site is selected from the group consisting of: a wound on the stem of a whole plant and a wound site on a root in culture.

4. A method of Claim 3 wherein the *Artemisia Annua* strain is selected from the group of strains consisting of: Yugoslavian, Walter Reed Army Institute of Research, West Virginia, American Arboretum and Super Annie.

5. A method of Claim 4 wherein the Ri plasmid DNA in the *Artemisia annua* produced is sufficient to cause hairy root formation when the resulting *Artemisia annua* is maintained under conditions appropriate for growth.

6. A method of Claim 4 wherein the *Agrobacterium rhizogenes* is *Agrobacterium rhizogenes* ATCC strain 15834.

7. A method of Claim 2 further comprising culturing *Artemisia annua* containing Ri plasmid DNA under conditions appropriate for hairy root growth, thereby producing *Artemisia annua* hairy roots.

8. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 1

9. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 2.

10. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 3.

11. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 4.

12. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 5.

13. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 6.

14. *Artemisia annua* containing Ri plasmid DNA produced by the method of Claim 7.

15. A method of producing *Artemisia annua* hairy roots comprising the steps of:

a) contacting a wound site on an *Artemisia annua* strain with *Agrobacterium rhizogenes* containing a Ri plasmid wherein the *Agrobacterium rhizogenes* is sufficient to cause hairy root formation in infected *Artemisia annua*, thereby producing *Artemisia annua* containing Ri plasmid DNA; and

b) maintaining *Art misia annua* containing Ri plasmid DNA under conditions appropriat for hairy root growth, thereby producing hairy roots from *Artemisia annua* containing Ri plasmid DNA.

16. A method of Claim 15 wherein the wound site is selected from the group consisting of: the stem of a whole plant and roots in culture.

17. A method of Claim 16 wherein the *Artemisia annua* strain is selected from the group of strains consisting of: Yugoslavian, Walter Reed Army Institute of Research, West Virginia, American Arboretum and Super Annie.

18. A method of Claim 17 wherein the Ri plasmid is the Ri plasmid contained in *Agrobacterium rhizogenes* ATCC strain 15834.

19. *Artemisia annua* hairy roots produced by the method of Claim 15.

20. *Artemisia annua* hairy roots produced by the method of Claim 16.

21. *Artemisia annua* hairy roots produced by the method of Claim 17.

22. *Artemisia annua* hairy roots produced by the method of Claim 18.

23. *Artemisia annua* hairy roots containing Ri plasmid DNA.

24. Art *misia annua* hairy roots of Claim 23, wherein the Ri plasmid is suffici nt to cause hairy root formation.

25. *Artemisia annua* hairy roots of Claim 24 wherein the Ri plasmid DNA is from the Ri plasmid present in *Agrobacterium rhizogenes* ATCC Strain 15834.

26. A method of obtaining enhanced amounts of artemisinin from transformed *Artemisia annua* hairy roots, comprising the steps of:

a) culturing *Agrobacterium rhizogenes* transformed *Artemisia annua* hairy roots under conditions appropriate for growth;

b) destabilizing *Artemisia annua* hairy root tissue membrane, thereby causing partial release of artemisinin and producing destabilized transformed *Artemisia annua* hairy root; and

c) removing artemisinin from the product of step (b);

d) restabilizing the destabilized transformed *Artemisia annua* root tissue membranes to substantially inhibit further release of artemisinin.

27. A method of Claim 26 wherein in step (b) destabilization is accomplished by exposing the plant tissue membrane to an elevated temperature for an appropriate period of time; wherein in step (c) the artemisinin is removed by contacting the transformed *Artemisia annua* hairy root with a nonaqueous solvent which does not significantly decrease plant tissue viability to the plant tissue surroundings and step (d) is accomplished by cooling the plant tissue for an appropriate period of time.

28. A method of Claim 27, wherein step (c) the nonaqueous solvent is selected from the group consisting of: ethanol, polyethylene glycol, poly-L-lysine, Chremaphor EL in a short chain alcohol, dimethylsulfoxide, Triton X-100, Brij, Tween-80 and cumene peroxide.

29. A method of obtaining partially purified artemisinin from plant tissue comprising the steps of:
(a) obtaining plant tissue containing artemisinin;
(b) grinding the plant tissue in the presence of ethanol thereby producing a mixture comprising cell debris and artemisinin in ethanol; and
(c) centrifuging the mixture produced in step (b) thereby producing a pellet containing cell debris and a supernatant containing partially purified artemesinin.

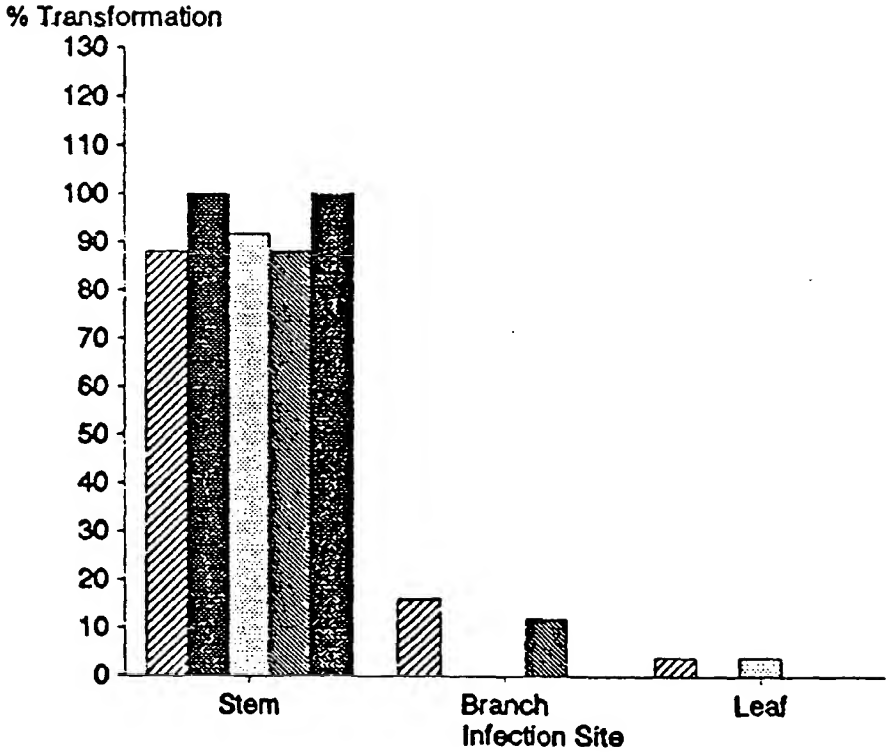

Figure 1

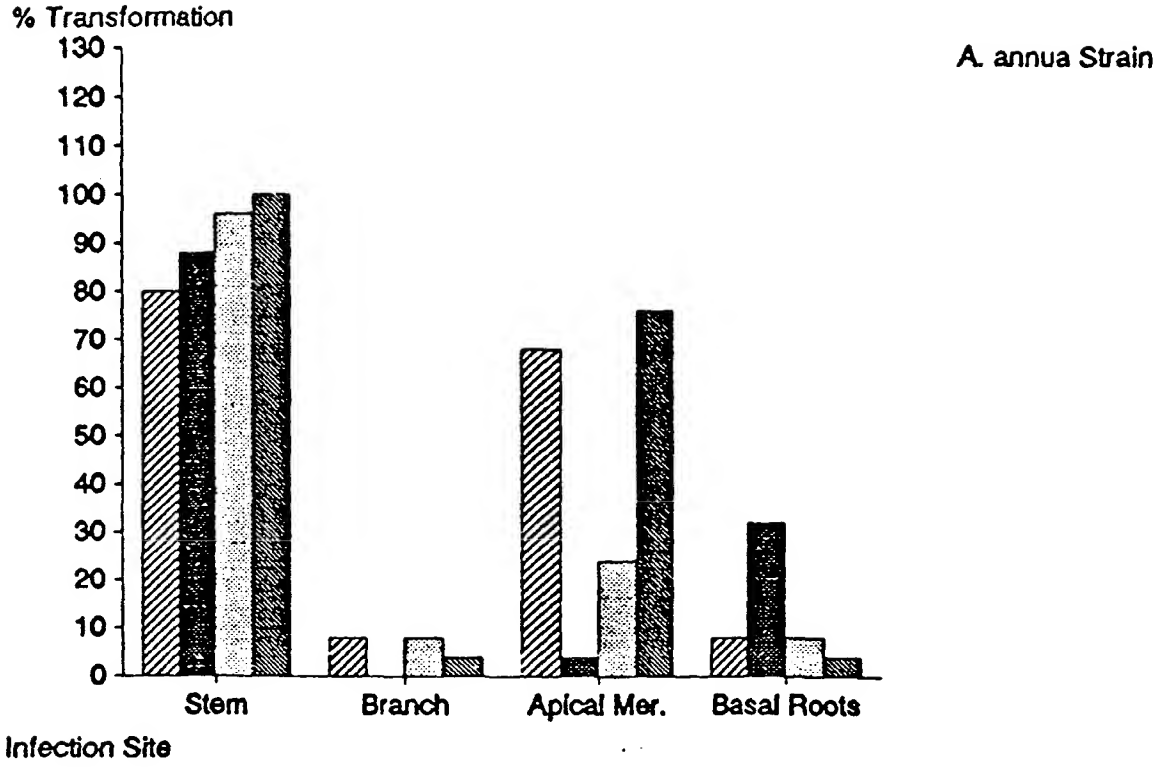

Figure 2

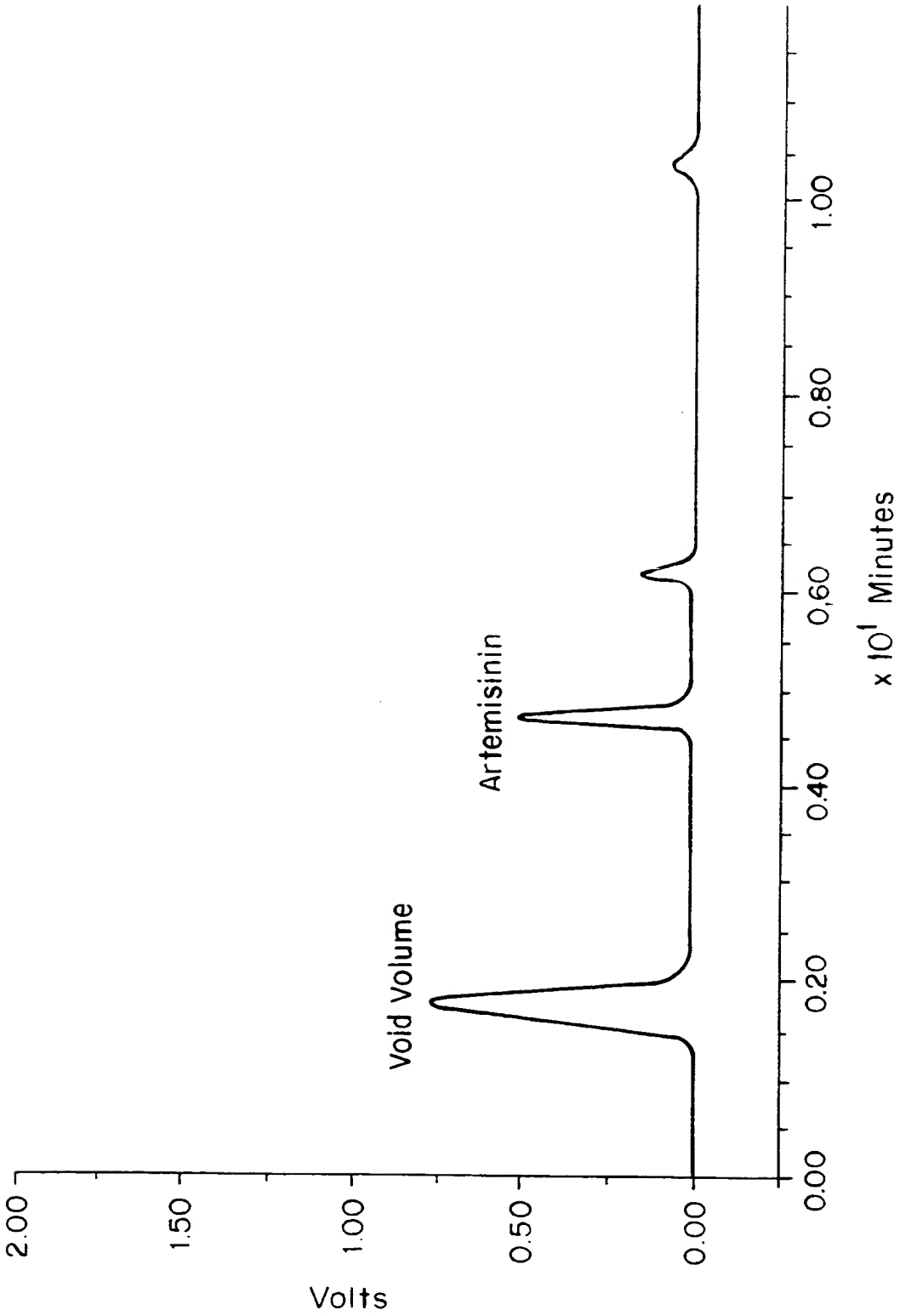

FIG. 3

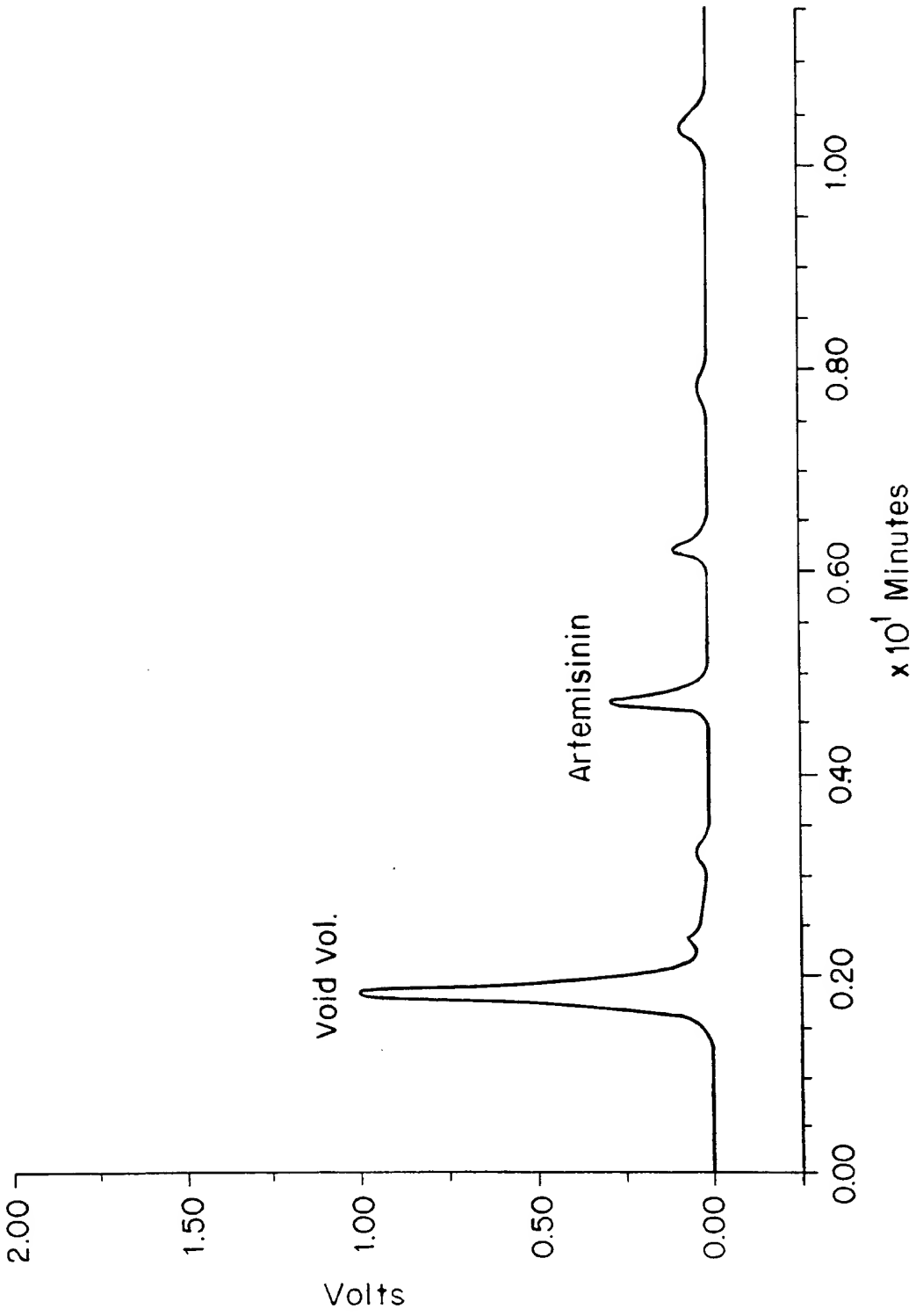

FIG. 4

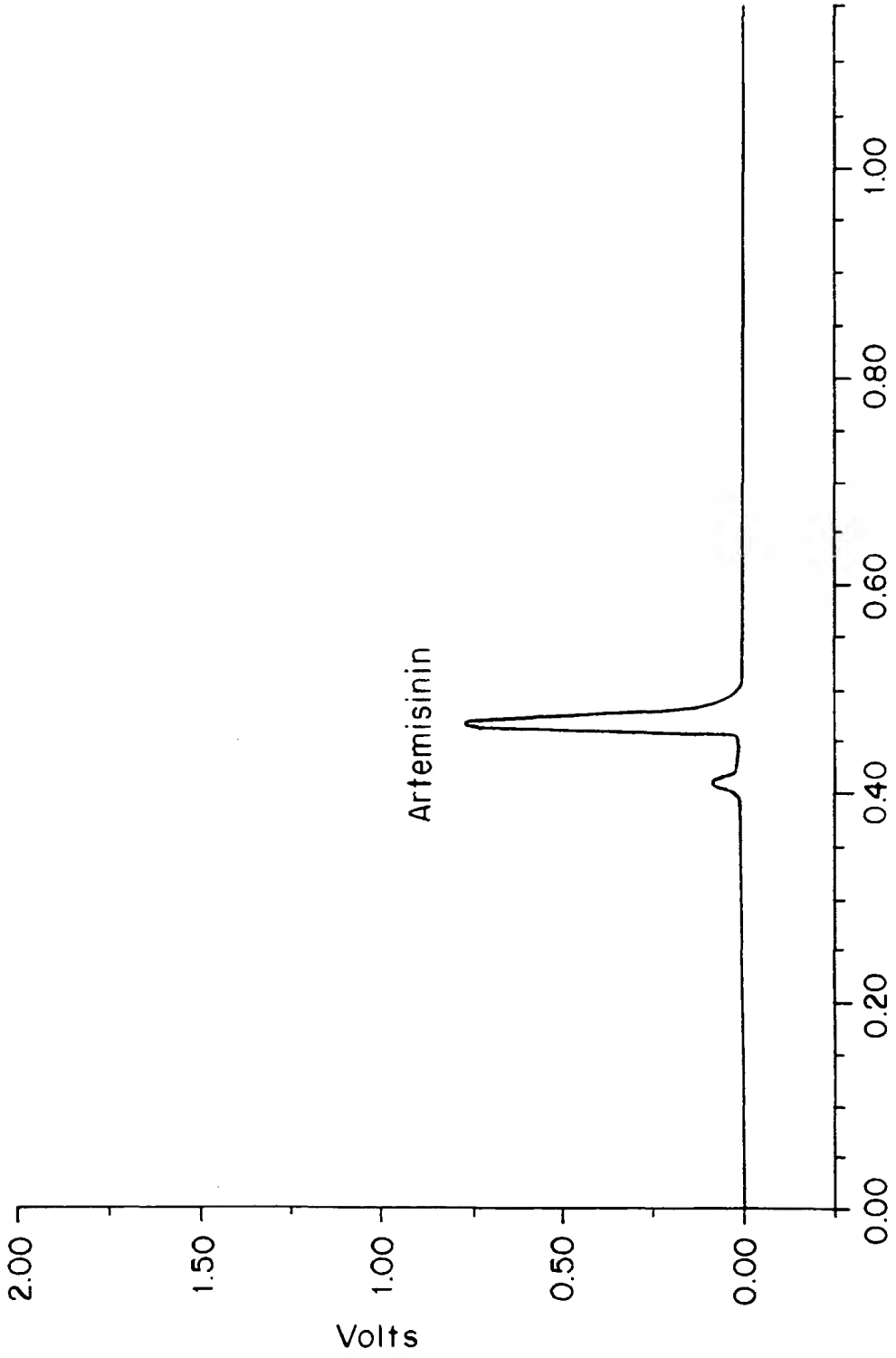

FIG. 5

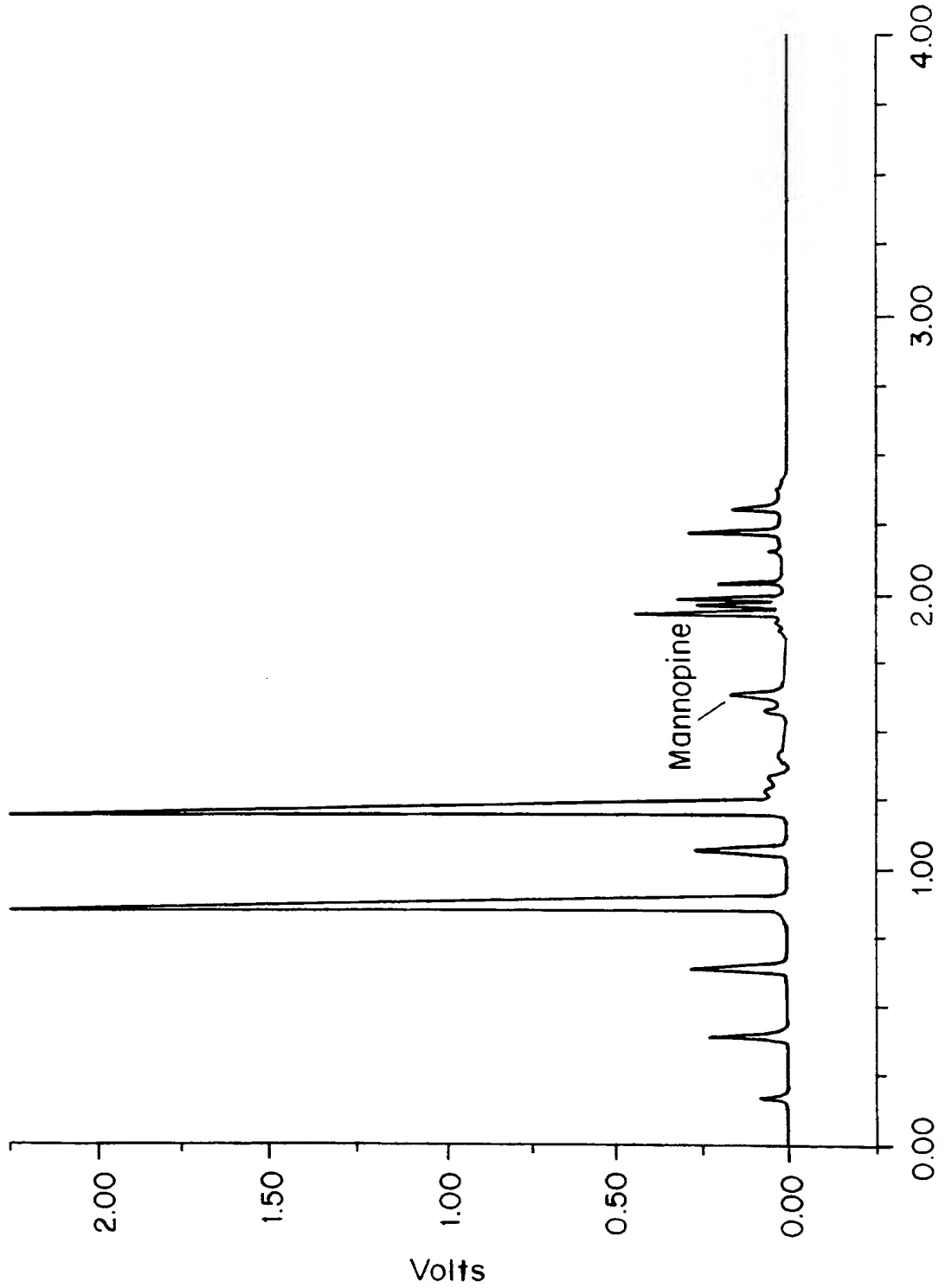

FIG. 6

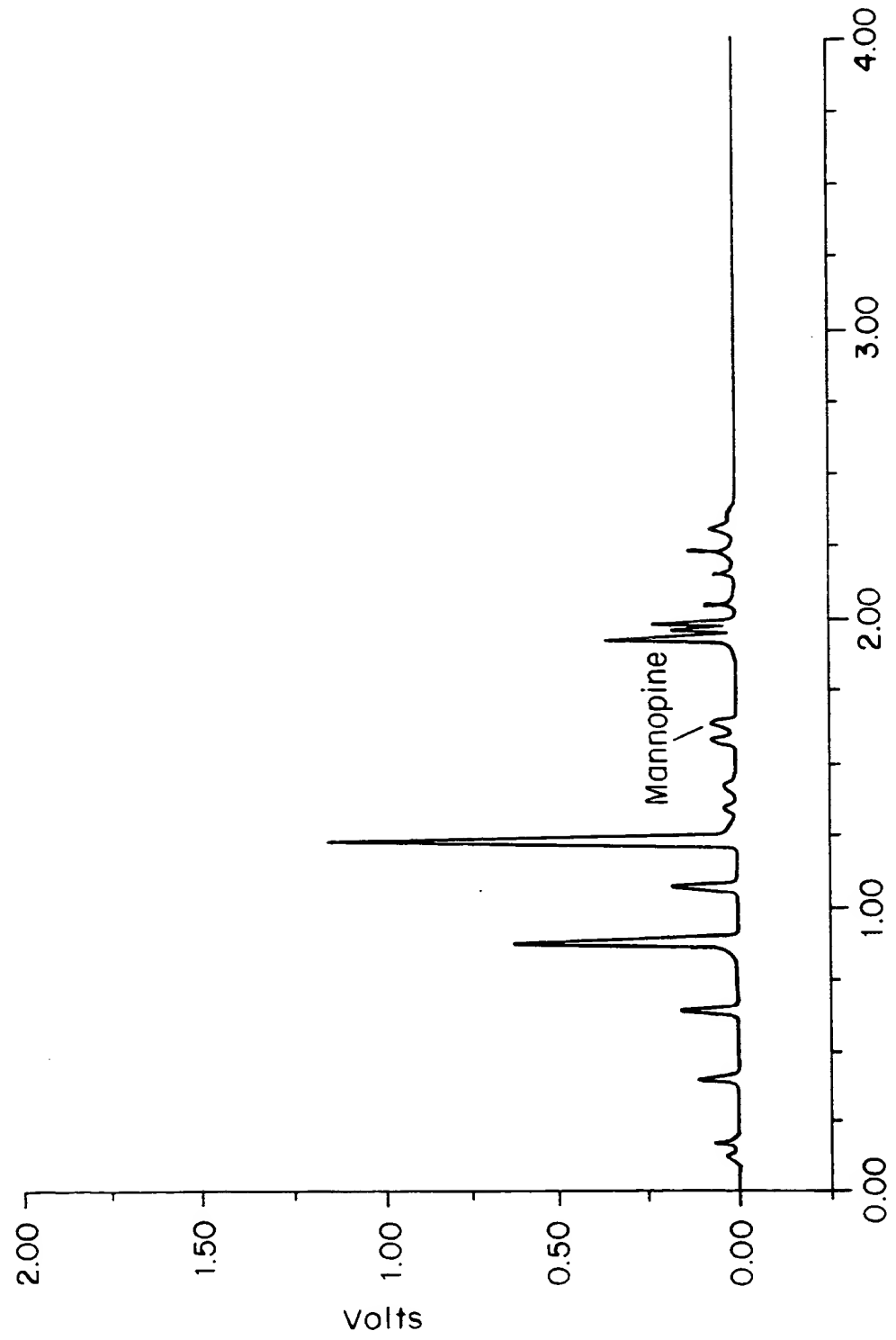

FIG. 7

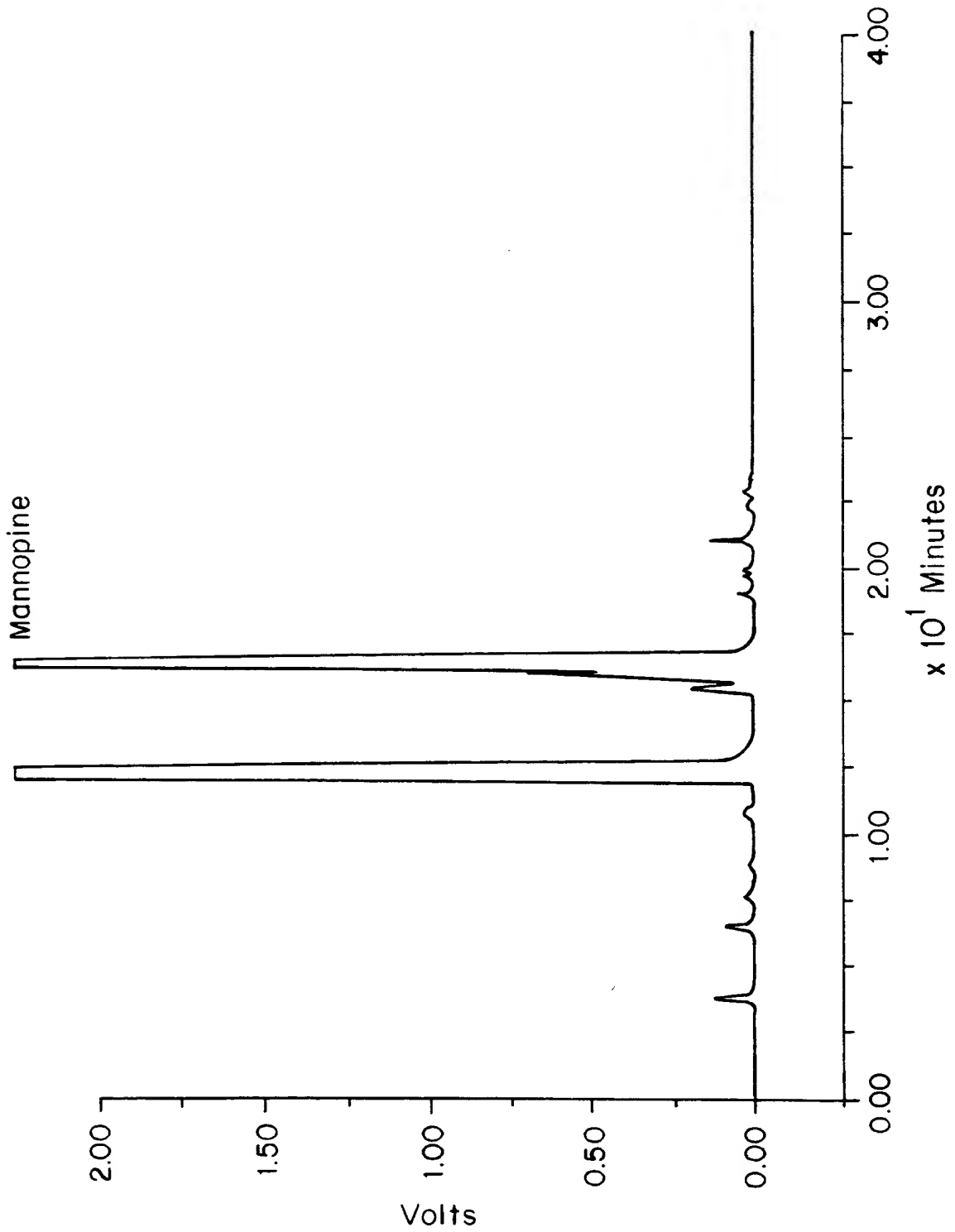

FIG. 8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/82, 5/04, 5/10
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(54) Title: ARTEMISIA ANNUA TRANSFORMED WITH AGROBACTERIUM RHIZOGENES

(57) Abstract

A method of transforming *Artemisia annua* with *Agrobacterium rhizogenes*. More particularly, a method for producing an *Agrobacterium rhyzogenes* hairy root cell culture of *Artemisia annua*. The method of transformation of *A. annua* includes the transformation of whole plants with *A. rhyzogenes*, and also includes transformation of *A. annua* root cultures with *A. rhizogenes*. The present invention also relates to transformed *A. annua*. The present invention also relates to a method for extracting enhanced amounts of a plant secondary metabolite from plant tissue with limited loss of tissue viability by reversibly permeabilizing the tissue membrane is disclosed.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/05950

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/82 C 12 N 5/04 C 12 N 5/10
C 12 P 17/18 C 07 D 307/92

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N C 12 P C 07 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	BIOESSAYS vol. 12, no. 1 , January 1990 pages 7 - 13 SIGNS, M.W., ET AL. 'The biosynthetic potential of plant roots' see page 9, line 44 - line 47 ---	1-25
Y	JOURNAL OF NATURAL PRODUCTS vol. 49, no. 3 , 1986 pages 504 - 507 NAIR, M.S.R., ET AL. 'Production of artemisinin in tissue cultures of Aretemisia annua' ---	1-25
A	see the whole document --- -/-	26-28

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26-10-1993	28 -02- 1994
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	A.D. MADDOX

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMISTRY AND BIOLOGY OF NATURALLY OCCURRING ACETYLENES AND RELATED COMPOUNDS 1988 , EDITED BY J. LAM, ET AL., ELSEVIER AMSTREDAM. pages 233 - 254 FLORES, H.E., ET AL. 'Production of polyacetylenes and thiopenes in heterotrophic and photosynthetic root cultures of Asteraceae' see page 235 - page 237 ---	1-28
A	BIOTECHNOLOGY vol. 5 , 1987 , NEW YORK US pages 800 - 804 HAMILL, J.D., ET AL. 'New routes to plant secondary products' see the whole document ---	1-28
A	JOURNAL OF BIOTECHNOLOGY vol. 10 , 1989 , AMSTERDAM NL pages 1 - 26 PARR, A.J. 'The production of secondary metabolites by plant cell cultures' see the whole document ---	1-28
A	US,A,4952603 (ELFERALY) 28 August 1990 see the whole document ---	1-28
P,X	ABSTRACTS OF PAPERS OF AMERICAN CHEMICAL SOCIETY vol. 205, no. 1-2 , 1993 page BIOT152 DIIORIO, A.A., ET AL. 'Modelling of hairy root growth' see abstract -----	1,5,8-14,19-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05950

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. CLAIMS 1-28
2. CLAIMS 29
   See PCT/ISA/206 mailed on 13/12/93.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   CLAIMS 1-28

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9305950 SA 76643

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/02/94
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4952603	28-08-90	None	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82